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| <b>(54) Title:</b> ANTISENSE OLIGONUCLEOTIDES TO SUPPRESS EICOSANOID FORMATION<br><br><b>(57) Abstract</b><br><br>The present invention provides new antisense oligonucleotides for the treatment of premature labor, premature rupture of the fetal membranes, premature cervical dilation and effacement, preeclampsia, endometriosis, rheumatoid arthritis, ARDs, and glomerulitis. The drugs are antisense oligonucleotides which attenuate the expression of either the mRNA encoding the cyclooxygenase protein or the mRNA encoding the thromboxane A <sub>2</sub> synthase protein. Once the mRNA encoding for cyclooxygenase is inhibited, the production of the cyclooxygenase is inhibited thereby inhibiting the production of the cyclooxygenase products such as prostaglandins and thromboxane. Thus, the antisense oligonucleotides provide novel therapy for the treatment of diseases involving cyclooxygenase products, prostaglandins and thromboxane metabolisms. Such diseases include immunological reproductive, cardiovascular, dermatologic, central nervous system disorders in which the release of cyclooxygenase products effects the genesis and progression of the disease. A second object of the invention is to provide new reagents for the research and study of the diseases involving cyclooxygenase products. |           |  |

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## ANTISENSE OLIGONUCLEOTIDES TO SUPPRESS EICOSANOID FORMATION

## BACKGROUND OF THE INVENTION

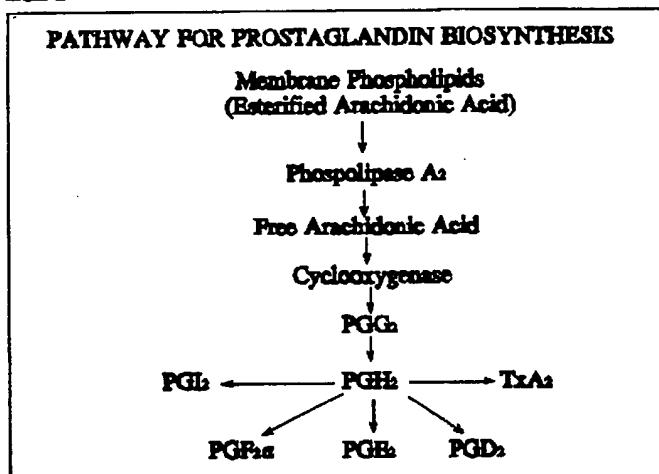
Prostaglandins are synthesized in mammals, including humans and are involved in inflammatory disease. Prostaglandins induce/contribute to a myriad of conditions, including the changes in vascular permeability leading to tissue edema and swelling, biochemical changes in the production of extracellular matrix degrading enzymes, such as fibroblast collagenase and elastase, fever and pain. Prostaglandins alter the contractile properties of vascular and non-vascular smooth muscle, leading to vasodilatation, vasoconstriction, uterine contractions, and bronchospasm.

The release of arachidonic acid from cellular membrane phospholipids and the subsequent production of eicosanoids such as cyclooxygenase products and lipoxygenase products is a hallmark feature of nearly all inflammatory diseases. Free arachidonic acid is the obligate precursor of cyclooxygenase, and its products. Under unstimulated cellular conditions, nearly all arachidonic acid is esterified in membrane phospholipids and is unavailable for eicosanoid biosynthesis. However, when a cell encounters certain extracellular stimuli, phospholipase A<sub>2</sub> cleaves arachidonic acid from the phospholipid, thereby permitting the arachidonic acid to be converted into prostaglandins by cyclooxygenase.

Once liberated, the bifunctional enzyme, cyclooxygenase, catalyzes the formation of prostaglandin (hereinafter "PGG<sub>2</sub>") by a bis-cyclooxygenation reaction. PGG<sub>2</sub> then undergoes a peroxidation reaction to form prostaglandin H<sub>2</sub> (hereinafter "PGH<sub>2</sub>") which is the immediate precursor for all subsequent prostaglandin synthetic reactions. In certain cell types, primarily platelets in which thromboxane (TxA<sub>2</sub>) is the major arachidonic acid metabolite, thromboxane synthase converts PGH<sub>2</sub> into thromboxane. Thromboxane is a potent vasoconstrictor and involved in vasoconstriction, coagulation, and preeclampsia. Other cell types, such as endothelial cells, synthesize mainly

prostacyclin ( $\text{PGI}_2$ ) which is produced from  $\text{PGH}_2$  via prostacyclin synthase.

Box 1



Prostagla  
ndin  $\text{E}_2$   
a n d

prostaglandin  $\text{F}_{2\alpha}$ , hereinafter " $\text{PGE}_2$ " and " $\text{PGF}_{2\alpha}$ " are the stimulators of uterine contractions and cervical dilatation and effacement which culminate in labor and delivery of the fetus at term. Keirse, M.J.N.C. "Eicosanoids in Human Pregnancy and Parturition", Eicosanoids in Reproduction, Mitchell, M.D., ed. 1990; CRC Press, Boca Raton, pp. 199-222.

The levels of  $\text{PGE}_2$  and  $\text{PGF}_{2\alpha}$  and their metabolites are also increased in the amniotic fluid of women in preterm labor with clinical signs of infection and are believed to be a causative factor of pre-term labor, Romero, R., Avila, C., and Sepulveda, W. "The Role of Systemic and Intrauterine Infection in Preterm Labor In: Preterm Birth: Causes, Prevention, and Management," 2d. ed., Fuchs, A.-R., Fuchs, F., and Stubblefield, P.G., eds., MacGraw-Hill, Inc., New York, 97-136. Preterm labor occurs in approximately 8-9% of all pregnancies, but accounts for 80% of perinatal morbidity and mortality in the United States. The cost of caring for premature infants who require long-term hospitalization can be as much as \$500,000 per infant. Moreover, many of these babies suffer long-range medical problems as a result of their prematurity.

Currently there are several categories of tocolytic agent for the treatment of premature uterine contractions. These drugs include  $\beta$ -sympathomimetics such as ritodrine and terbutaline,

calcium channel antagonists and the prostaglandin synthase inhibitor indomethacin. The  $\beta$ -sympathomimetic agents and calcium channel blockers have proven only partially successful in arresting uterine activity in women who are in latter stages of preterm labor. Moreover, these drugs produce maternal side-effects such as tachycardia, agitation, increased cardiac output, increased plasma volume, and even a small number of maternal fatalities. In women with contraindications to the use of  $\beta$ -sympathomimetics, there are often no alternative therapies to offer for the treatment of preterm labor. In addition, these drugs have been associated with fetal tachycardia and other potentially harmful fetal side-effects, such as intracerebral hemorrhage. Calcium channel blockers such as nifedipine and magnesium sulfate have shown some efficacy, but also have shown untoward maternal and fetal side-effects, [Vanden Veyuer, I. and Moisek.] "Prostaglandin Synthetase Inhibitors in Pregnancy," Obstetrical and Gynecological Survey, 1993; 48: 493-502; Eronen, M. et al., "The Effects of Indomethacin and a  $\beta$ -Sympathomimetic Agent on the Fetal Ductus Arteriosus During Treatment of Premature Labor: A Randomized Double-Blind Study," Am. J. Obstet. Gynecol., 1991; 164: 141-146.

Indomethacin, an irreversible inhibitor of cyclooxygenase, and thus a prostaglandin synthesis inhibitor, has shown promise in the arrest of uterine contractions. However, indomethacin readily crosses the placental barrier, and causes undesirable side-effects in the fetus, including constriction of the ductus arteriosus and pulmonary hypertension, and altered cerebral blood flow. Gamissans, O. and Balasch, J. "Prostaglandin Synthetase Inhibitors in the Treatment of Preterm Birth," Preterm Birth: Causes, Prevention, and Management, 2d. ed., Fuchs, A.-R., Fuchs, F., and Stubblefield, P.G., eds., MacGraw-Hill, Inc., New York, 309-332.

Thus, while a few traditional pharmacologic agents exist for the treatment of premature labor in women, each has maternal and/or fetal side-effects which significantly limit their usefulness. The obstetrician is faced with the task of attempting to combat preterm uterine contractions with inadequate therapeutic tools.

Preterm premature rupture of the fetal membranes also referred to as "PROM" (that is, the bag of waters surrounding the fetus) and premature biochemical changes, such as premature dilatation and effacement also occur with preterm labor, and usually lead to premature expulsion of the fetus. These events are also mediated by prostaglandins, specifically PGE<sub>2</sub>. Rath, W. et al., "Biochemical Changes in Human Cervical Connective Tissue After Intracervical Application of Prostaglandin E<sub>2</sub>," Prostaglandins 1993; 45: 375-380. Currently, there is no pharmacologic treatment available for the treatment of premature cervical dilatation or PROM. Once the fetal membranes are ruptured, labor and delivery of the fetus are usually inevitable. However, if PROM occurs prior to 26-27 weeks gestation, the fetus is rarely able to survive. The only current treatment for premature dilatation and effacement is surgical ligation of the cervical canal. Yet the ligation itself often leads to uterine activity and preterm delivery.

Preeclampsia, a disease associated with an increased production of TXA<sub>2</sub> and a shift in the ratio of PGI<sub>2</sub> to TXA<sub>2</sub>, accounts for approximately 7% of all first pregnancies. Friedman, S.A., "Preeclampsia: A Review of the Role of Prostaglandins," Obstet. Gynecol., 1988; 71: 122-137. Zeeman, G.G. and Dekker, G.A., "Pathogenesis of Preeclampsia: A Hypothesis," Clin. Obstet. Gynecol., 1992; 35: 317-337. Preeclampsia is characterized by maternal hypertension, renal impairment including proteinuria, liver damage, systemic vasospasm, hypercoagulation and edema, and in severe cases, seizures and even death. Preeclampsia is the leading cause of maternal mortality in most developed nations. Preeclampsia also causes reduced uteroplacental blood flow and restricts the transfer of nutrients to the fetus. This leads to intrauterine growth retardation, fetal compromise, and often necessitates preterm delivery of the infant, which contributes to the high premature birth rate. Unfortunately, if the disease occurs during the late second or early third trimester, the fetus is too premature to survive outside the uterus. The mortality rate of these infants is extremely high.

There are very few treatments available for preeclampsia. Recently, low-dose aspirin has been used for the treatment of women who are at increased risk for developing preeclampsia. However, aspirin has not been effective at alleviating the disease symptoms once they occur. Thus, at present no palliative therapies exist for the treatment of this pregnancy complication.

Endometriosis, a condition in which the uterine lining proliferates and tissue escapes into the peritoneal cavity, is a painful and debilitating disorder which can require surgical correction and often threatens the reproductive ability of the patient. At present, hormonal therapy is used to inhibit uterine endometrial proliferation. In more severe cases, excess tissue is removed surgically. However, neither of these treatments relieve the painful effects of excess prostaglandin production by the endometrial tissues.

Rheumatoid arthritis is a chronic inflammatory disease localized to joint surfaces and characterized by synovial fibroblast proliferation, degradation of bone extracellular matrix, joint swelling and crippling pain. Lefer, A.M., "Eicosanoids as Mediators of Ischemia and Shock," Fed. Proc., 1985; 44: 275-280. PGE<sub>2</sub> has been shown to stimulate the production of collagenase by isolated synoviocytes and elicits bone matrix degradation by osteoclasts. Synovial cells isolated from rheumatoid arthritis patients produce approximately ten times more PGE<sub>2</sub> than cells from normal patients. Current therapies to treat rheumatoid arthritis rely on NSAIDS such as aspirin, ibuprofen and indomethacin and local administration of glucocorticoids such as dexamethasone and hydrocortisone to reduce joint pain and swelling. However, these agents cause several side-effects, chiefly gastric upset. Long-term glucocorticoid use causes liver and cardiovascular damage and loss of bone mass.

Adult respiratory distress syndrome (ARDS) and shock is a rare, but life threatening condition which can be precipitated by severe systemic infection, traumatic injury or shock and is characterized by acute endothelial cell damage in the lung, and in severe cases respiratory collapse and death. Proinflammatory cytokines, for example, interleukin-1, and tumor necrosis

factor- $\alpha$ , (TNF- $\alpha$ ) are thought to be the primary mediators of ARDS and they are proposed to act via arachidonic acid metabolites and platelet-activating factor. PGI<sub>2</sub> and PGE<sub>2</sub> cause increased vascular permeability and interstitial edema within the lung parenchyma and accumulation of extravasated fluid and proteins in the alveolar space, while PGF<sub>2 $\alpha$</sub>  and thromboxane cause vasoconstriction and pulmonary hypertension. PGE<sub>2</sub> is also a modulator of neutrophil and monocyte chemotaxis to the lung. In addition, these patients often develop systemic hypotension, leading to compromise of multiple organ systems. Thromboxane also leads to platelet activation and adhesion to microvascular wall, precipitating thrombosis and ischemia. Animal models of ARDS have provided evidence that cyclooxygenase inhibitors may attenuate some of the clinical manifestations of ARDS. However, since the production of prostaglandins in this disease is progressive, it is clinically difficult to use conventional anti-cyclooxygenase agents for the effective treatment of ARDS.

Glomerulonephritis is an inflammatory disease of the kidney which results in the influx of inflammatory cells, such as neutrophils and monocytes into the renal parenchyma. Macrophages which migrate to the kidney from distal sites, release a myriad of cytokines and eicosanoids, for example, TxA<sub>2</sub>, leukotriene B<sub>4</sub> resulting in reduced renal blood flow. Since TxA<sub>2</sub> released by inflammatory macrophages is very deleterious to normal renal function, chronic injury may occur, ultimately leading to complete renal failure. In such cases, the patient is faced with certain long-term renal dialysis and even kidney transplantation. Traditional anti-inflammatory drugs such as non-specific anti-inflammatory drugs and glucocorticoids are usually not suitable since they cause generalized inhibition of prostaglandin biosynthesis, including inhibition of PGE<sub>2</sub> which is required for normal renal physiologic function. Lianos, E.A., "Eicosanoid Biosynthesis and Role in Renal Immune Injury," Prostaglandins Leukot. Essent. Fatty Acids, 1990; 41:1-12.

It would be desirable to have drugs for the premature labor, PROM, premature effacement and dilation, endometriosis, rheumatoid arthritis, ARDS and glomerulitis, that would eliminate



the condition, without the side effects of conventional treatments.

#### SUMMARY OF THE INVENTION

The present invention provides new antisense oligonucleotides for the treatment of premature labor, premature rupture of the fetal membranes, premature dilation and effacement, preeclampsia, endometriosis, rheumatoid arthritis, ARDs, and glomerulitis. The antisense oligonucleotides which attenuate the expression of either the mRNA encoding the cyclooxygenase protein or the mRNA encoding the thromboxane A<sub>2</sub> synthetase protein. Once the mRNA encoding for cyclooxygenase is inhibited, the production of cyclooxygenase is inhibited thereby inhibiting the production of the cyclooxygenase products such as prostaglandins and thromboxane. The antisense oligonucleotide which only alleviates the production of mRNA encoding thromboxane A<sub>2</sub> synthase inhibits the production of thromboxane. Az synthetase thereby inhibiting the production of thromboxane. As a result, the production of prostaglandins is not inhibited. Thus, the antisense oligonucleotides provide novel therapy for the treatment of diseases involving prostaglandins and thromboxane. Such diseases include immunological, reproductive, cardiovascular, dermatologic, and central nervous system disorders in which the release of cyclooxygenase products effects the genesis and progression of the disease. A second object of the invention is to provide new reagents for the research and study of the diseases involving cyclooxygenase products.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a northern blot showing the COX-2 in RNA expression in mouse 3T3 cells: treated with PMA (lane 2); preincubated with 5  $\mu$ M antisense S-oligonucleotide mCOX-2.2 and treated with PMA (lane 4); preincubated with 5  $\mu$ M antisense S-oligonucleotide mCOX-2.2 and no PMA (lane 3); preincubated with 10  $\mu$ M antisense S-oligonucleotide mCOX-2.2 and treated with PMA (lane 6); preincubated with 10  $\mu$ M antisense S-oligonucleotide

mCOX-2.2 and no PMA (lane 5); and not treated with either PMA or the S-oligonucleotide (lane 1).

Figure 2. is a graph demonstrating mCOX-2.4 antisense oligonucleotide suppression of PGE<sub>2</sub> production in a dose-dependent manner. The cells were preincubated for 18 hours with either 5 or 10  $\mu$ M of S-oligonucleotide corresponding to the mCOX-2.4 sequence. Cells challenged with PMA are represented by cross-hatched bars. Cells not challenged with PMA are represented by open bars. The data are mean $\pm$ SEM of 4 replicates per condition.

Figure 3. is a graph demonstrating mCOX-2.2, mCOX-2.3, and mCOX-2.4 antisense S-oligonucleotides suppression of PGE<sub>2</sub> production in PMA stimulated 3T3 cells. The cells challenged with PMA are represented by closed and cross-hatched bars. Cells not challenged with PMA are represented by open bars. The data are mean $\pm$ SEM of 8 replicates per condition.

Figure 4. is a graph demonstrating mCOX-2.4 antisense oligonucleotides attenuating PGE<sub>2</sub> production in stimulated mouse 3T3 cells. Cells were preincubated for 18 hours with 10  $\mu$ M of either N-oligonucleotide or S-oligonucleotide corresponding to the mCOX-2.4 sequence. Cells challenged with PMA are represented by cross-hatched bars. Cells not challenged with PMA are represented by open bars. The data are the mean $\pm$ SEM of 4 replicates per condition and are representative of 2 experiments.

Figure 5. is a graph demonstrating hCOX-2.1, hCOX-2.2, hCOX-2.8, and hCOX-2.9 antisense S-oligonucleotide suppression of PGE<sub>2</sub> production in PMA simulated WISH cells. The cells challenged with PMA are represented by solid bars and by cross-hatched bars. Cells not challenged with PMA are represented by open bars. The data are mean $\pm$ SEM of 4-8 replicates per condition.

Figure 6. is a graph demonstrating hCOX-2.9 antisense S-oligonucleotide suppression of PGE<sub>2</sub> production in a dose-dependent manner in WISH cells. Cells challenged with interleukin-1 $\beta$  are represented by closed bars. Cells not challenged with interleukin-1 $\beta$  are represented by open bars. The data are mean $\pm$ SEM of 4-8 replicates per condition and are representative of 2 experiments.

Figure 7 is a western blot showing the COX protein expression in WISH cells: treated with interleukin-1 $\beta$  (lane 2); preincubated with 10  $\mu$ M antisense S-oligonucleotide hCOX-2.1 and treated with interleukin-1 $\beta$  (lanes 3-9); and not treated with either interleukin-1 $\beta$  or S-oligonucleotide (lane 1).

Figure 8. is a graph demonstrating hCOX-1.1 antisense S-oligonucleotide suppression of PGE<sub>2</sub> production in WISH cells and in HES cells. Cells not challenged with interleukin-1 $\beta$  are represented by open bars and cells challenged with interleukin 1 $\beta$  are represented by cross-hatched bars. The data are mean $\pm$ SEM of 4 replicates per condition.

Figure 9 is a western blot showing the COX-2 protein expression in wish cells: treated with interleukin-1 $\beta$  (lane 2); preincubated with 5  $\mu$ M antisense S-oligonucleotide hCOX-2.1 and treated with interleukin-1 $\beta$  (lane 3); preincubated with 10  $\mu$ M antisense S-oligonucleotide hCOX-2.1 and treated with interleukin-1 $\beta$  (lane 4); preincubated with 20  $\mu$ M S-oligonucleotide hCOX-2.1 and treated with interleukin-1 $\beta$  (lane 5); preincubated with 40  $\mu$ M S-oligonucleotide hCOX-2.1 and treated with interleukin 1 $\beta$ ; and not treated with either interleukin-1 $\beta$  or the S-oligonucleotide (lane 1).

Figure 10 is a northern blot showing the COX2 mRNA expression in mouse macrophages: treated with LPS (lane 2); preincubated with 10  $\mu$ M antisense S-oligonucleotide mCOX-2.2 and treated with LPS (lane 3); and not treated with either LPS or the S-oligonucleotide (lane 1).

Figure 11. is a graph demonstrating hTXS antisense S-oligonucleotide suppression of thromboxane production in human ED<sub>27</sub> cells.

#### DETAILED DESCRIPTION OF THE INVENTION

This invention provides antisense oligonucleotides for the treatment, diagnosis and research of diseases which involve the production and release of metabolites of arachidonic acid, including prostaglandins, thromboxane and prostacyclin. The antisense oligonucleotides are complementary to, and bind the messenger ribonucleic acids (mRNAs) which encode for either: the thromboxane A synthase; or the constitutive cyclooxygenase,

referred to herein as "COX-1"; or the inducible cyclooxygenase, referred to herein as "COX-2". Several of the antisense oligonucleotides inhibit the expression of mRNAs encoding for cyclooxygenase, either COX-1 or COX-2. Suppressing the expression of the mRNA which encodes cyclooxygenase prevents the production of cyclooxygenase thereby preventing the generation of prostaglandins and thromboxane which are synthesized by the cyclooxygenase. The antisense nucleotides of the present invention do not suppress lipoxygenase products, only cyclooxygenase products. Completely or even partially suppressing the production of cyclooxygenase and the resulting metabolites is useful as a palliative therapy for the treatment of diseases in which arachidonic acid release and the production of cyclooxygenase products such as PGE<sub>2</sub>, PGF<sub>2α</sub>, PGD<sub>2</sub>, PGI<sub>2</sub>, and thromboxane are part of the disease pathophysiology.

The antisense oligonucleotides directed to the mRNA encoding thromboxane synthase selectively suppress the production of thromboxane, while sparing other physiologically important prostaglandins. Such oligonucleotides offer a palliative treatment of glomerulonephritis by specific blockade of thromboxane release.

#### Antisense Oligodeoxynucleotides

The antisense oligonucleotides which are directed against the mRNA of human thromboxane A<sub>2</sub> synthase and both the constitutive and inducible forms of murine and human cyclooxygenases, were constructed from the corresponding cDNA sequences. The cDNA sequences listed in Table 1 were obtained from Genbank, an on-line computer service. Then the nucleotide sequences of the cDNAs were fed into by a microcomputer. The regions of each mRNA molecule which would provide a theoretically stable hybrid with the cognate antisense oligodeoxynucleotide were chosen based upon thermodynamic conditions using algorithms in the software OLIGO® available from National Biosciences in Minneapolis, MN. Most of the antisense oligonucleotides contain 18 deoxynucleotides. The 18 deoxynucleotides are the preferred length in terms of stability and efficiency of uptake into cells. However, oligonucleotides containing for example, from 12 to 15 nucleotides or longer oligonucleotides, containing, for example,

20 nucleotides are also suitable. Such oligonucleotides are shown in Tables 2 and 3. The antisense oligonucleotides of the present invention are all deoxynucleotides.

Previous studies using many different mRNA targets have shown that the regions of the mRNA to which antisense oligonucleotides hybridize are critical for efficient inhibition of the desired function. Thus, the 5'-untranslated region just upstream from the ATG start codon and the 3'-untranslated region were the predominant targets for the antisense oligonucleotides. Once antisense oligonucleotides that provided thermodynamically stable hybrids with the target mRNA were designed, they and the phosphorothioate derivatives of such oligonucleotides were ordered from *Oligos Etc.*®, Minneapolis, MN, which produced the desired oligonucleotides according to conventional phosphoramidite chemistry. The oligonucleotides that were unmodified, are hereinafter also referred to as "N-oligonucleotides". The phosphorothioate derivative nucleotides are hereinafter also referred to as "S-oligonucleotides".

Table 1

Description of cDNA Sequences Used to Construct  
Antisense S-oligodeoxynucleotides

| Target mRNA | GenBank Accession # | cDNA Length | Coding Sequence | 5'-UTR Length | 3'-UTR Length |
|-------------|---------------------|-------------|-----------------|---------------|---------------|
| mCOX-1      | M34141              | 2757        | 37-1844         | 1-36          | 1843-2757     |
| mCOX-2      | M64291              | 3986        | 125-1939        | 1-124         | 1940-3986     |
| hCOX-1      | M59979              | 2554        | 6-1805          | 1-5           | 1806-2554     |
| hCOX-2      | M90100              | 3387        | 98-1912         | 1-97          | 1913-3387     |
| hTXS        | M80646              | 1719        | 172-1554        | 1-171         | 1555-1719     |

mCOX-1 and mCOX-2 refer to murine COX-1 and COX-2.  
hCOX and hCOX-2 refer to human COX-1 and COX-2.

Table 2

## Mouse Cyclooxygenase-2 Antisense S-oligodeoxynucleotides

| Oligo. Designation | Nucleo. Length | Start Position | Oligo. Sequence           |
|--------------------|----------------|----------------|---------------------------|
| mCOX-1.1           | 18             | 13             | 5'-GGGAGTGGATGGATGTGC-3'  |
| mCOX-1.2           | 18             | 1864           | 5'-AAAACCTCCTCCCTCCAGA-3' |
| mCOX-1.3           | 18             | 1904           | 5'-CCACGAAAACCCACATCA-3'  |
| mCOX-1.4           | 18             | 1987           | 5'-CCGAAAGTGGCAAAATCA-3'  |
| mCOX-1.5           | 18             | 16             | 5'-TCTGGGAGTGGATGGATG-3'  |
| mCOX-2.2           | 18             | 104            | 5'-AGAGGTGGCAGCGGAGGT-3'  |
| mCOX-2.3           | 18             | 2126           | 5'-AGAGGAATCAATGCTGAT-3'  |
| mCOX-2.4           | 18             | 112            | 5'-GAGCATCGCAGAGGTGGC-3'  |
| mCOX-2.5           | 18             | 2371           | 5'-TTGGACCCCTTGTGTTGA-3'  |

Length of antisense S-oligodeoxynucleotides is in bases. Start position denotes the position of the first base of the antisense oligonucleotide within the cDNA sequence.

Table 3

## Human Cyclooxygenase-2 Antisense S-oligodeoxynucleotides

| Antisense Oligo. Designation | Nucleo. Length | Start Position | Oligo. Sequence           |
|------------------------------|----------------|----------------|---------------------------|
| hCOX-1.1                     | 18             | 1              | 5'-GACTCCGGCTCATGGCGC-3'  |
| hCOX-2.1                     | 18             | 51             | 5'-GTAGGCTTTGCTGTCTGA-3'  |
| hCOX-2.2                     | 12             | 97             | 5'-CGGGCGAGCATC-3'        |
| hCOX-2.3                     | 18             | 79             | 5'-GCAGCGGCGGGCAGGGCG-3'  |
| hCOX-2.4                     | 15             | 89             | 5'-GAGCATCGCAGCGGC-3'     |
| hCOX-2.5                     | 18             | 89             | 5'-GGCGAGCATCGCAGCGGC-3'  |
| hCOX-2.6                     | 15             | 97             | 5'-GCGCGGGCGAGCATC-3'     |
| hCOX-2.7                     | 18             | 97             | 5'-AGGGCGCGGGCGAGCATC-3'  |
| hCOX-2.8                     | 18             | 2037           | 5'-ATGACTCCTTTCTCCGCA-3'  |
| hCOX-2.9                     | 20             | 2789           | 5'-TTTTGGCGGGTTATGGGGT-3' |

Length of antisense S-oligonucleotides is in bases. Start position denotes the position of the first base of the antisense oligonucleotide within the cDNA sequence.

Table 4

Human Thromboxane A<sub>2</sub> Synthase Antisense  
S-oligodeoxynucleotides

| Oligo.<br>Designation | Length<br>(nucleotide) | Start<br>Position | Nucleotide Sequence      |
|-----------------------|------------------------|-------------------|--------------------------|
| hTXS-1                | 18                     | 54                | 5'-AACCAAGCAAACATCACA-3' |
| hTXS-2                | 18                     | 94                | 5'-TTATGGGAACCGTGCTCT-3' |
| hTXS-3                | 18                     | 165               | 5'-GCTTCCATCATTCCTCTG-3' |
| hTXS-4                | 18                     | 1554              | 5'-TAGGGCAGATTTGGATTC-3' |
| hTXS-5                | 18                     | 1701              | 5'-GGCTTTCATCACTTCAG-3'  |
| hTXS-6                | 18                     | 166               | 5'-GGCTTCCATCATTTCTCT-3' |

When received from the commercial assembler, the antisense oligonucleotides were reconstituted in sterile distilled water from lyophilized stocks at working concentration of 2 mM. Since the antisense oligonucleotides were evaluated in cell cultures, the working solutions of the antisense oligonucleotide were diluted further with serum-free culture media for addition to cell cultures. In some cases, the antisense nucleotides were added to cell cultures which had been preincubated with 10 µg/ml of cationic liposomes *Lipofection reagent*®, available from GIBCO®/BRL, Bethesda, MD to enhance cellular uptake. However, addition of the cationic liposomes was found to be unnecessary under most circumstances and an 18 hour preincubation with the S-oligonucleotide prior to stimulation of prostaglandin formation was sufficient for uptake of the oligonucleotides. After the cell cultures were incubated with the S-oligonucleotide being evaluated, cell cultures were stimulated with an agent to promote the production of the thromboxane or the prostaglandin or both. The effect of the antisense oligonucleotides were evaluated in

several different ways. At the end of each stimulation period, the media were collected from the cells and the thromboxane or the prostaglandin or both, were measured by specific radioimmunoassay. The antisera and protocol for these assays have been described in Fertel et al., 1981, Biochem. Biophys. Res. Comm., 102: 1028-1033; Kniss et al., 1992, Prostaglandins, 44: 237-244; Kniss et al., 1993, Prostaglandins, 45: 27-33. The COX mRNA was determined by preparing northern blots by harvesting the cells, extracting the total mRNA, fractionating the RNA on a 1% agarose gel, blotting the appropriate fraction onto nylon membranes, probing the blots with a [<sup>32</sup>P] labelled cDNA probe encoding the COX cDNA. Alternatively, the proteins were extracted from cells with 1% Triton X-100, and western blots were prepared by fractionating the proteins on a 10% polyacrylamide gel, transferring to nitrocellulose and then probing with an antibody specific for enzyme being measured. The probe was then detected by standard methods.

#### THE ANTISENSE OLIGONUCLEOTIDES AS CYCLOOXYGENASE INHIBITORS

##### Inhibition of Cyclooxygenase in Fibroblasts

Mouse NIH 3T3 cells (fibroblasts) were used as a model system for the evaluation of the antisense oligonucleotides on prostaglandin production by fibroblasts. Prostaglandin is formed by fibroblasts isolated from a variety of tissue sources and disease processes, for example, synovial cells from rheumatoid arthritis patients, interstitial fibroblasts from patients with adult respiratory distress syndrome [ARDS], cervical incompetence. The 3T3 cells are an immortalized population of cells which manifest many properties of mouse



fibroblasts maintained in primary culture. To establish a culture of 3T3 cells, they were seeded into 48-well plates at a concentration of  $2 \times 10^5$ /well which had been precoated with type I collagen, in Dulbecco's Modified Eagle Media/Ham's F12+10% newborn calf serum and grown at 37°C in 5% CO<sub>2</sub>/95% air.

To evaluate the antisense oligonucleotides for either prostaglandin suppression or mCOX mRNA suppression, the 3T3 cells were seeded into 35 mm dishes ( $2 \times 10^6$ /dish) in Ham's F12/Dulbecco's Modified Eagle Media supplemented with 10% newborn calf serum. After 24 hours, the cells were incubated in 0.25 serum-free medium for 18 hours with the desired concentration of the desired mCOX antisense S-oligonucleotide. Control cells received vehicle only during the preincubation. The cells were then challenged with 30 nM phorbol 12-myristate 13-acetate, hereinafter also referred to as "PMA", for 4 hours. PMA is an agent known to promote prostaglandin formation.

To determine the effect of the antisense S-oligonucleotide mCOX-2.2 on the COX-2 mRNA, the 3T3 cells were incubated in 0.25 serum-free medium for 18 hours with either 5 or 10  $\mu$ M antisense S-oligonucleotide mCOX-2.2. Total RNA was prepared, fractionated on a 1% agarose gel, and blotted onto nylon membranes. The blots were probed with a [<sup>32</sup>P] labelled cDNA probe encoding the murine COX-2 cDNA. The TIS10 probe was donated by Dr. Harvey Herschman at UCLA. The Northern blot is shown in Figure 1.

As shown, lanes 5 and 6 of Figure 1, 10  $\mu$ M of the antisense S-oligonucleotide mCOX-2.2 significantly suppressed the formation of the mRNA encoding COX-2. Moreover, there is a dose-dependent diminution in COX-2 mRNA expression (arrowhead) in cells treated with 5  $\mu$ M of the antisense S-oligonucleotide mCOX-2.2, shown in

lanes 3 and 4, or 10  $\mu\text{M}$  S-oligonucleotide shown in lanes 5 and 6, as compared to control cells which are shown in lane 2. Lanes 3 and 5 received only the S-oligonucleotide and no PMA, that is they were not stimulated; therefore, no expression of COX-2 mRNA was expected in these cells.

The antisense S-oligonucleotide mCOX-2.4 was evaluated for suppressing prostaglandin production in the same culture system as described above. Either 5  $\mu\text{M}$  or 10  $\mu\text{M}$  of the antisense S-oligonucleotide, mCOX-2.4 was added to the cultures. PGE<sub>2</sub> production was measured by specific radioimmunoassay.

As shown in Figure 2, the 10  $\mu\text{M}$  dosage significantly suppressed the production of PGE<sub>2</sub>. Again, a dose-dependent relationship was established; the 5  $\mu\text{M}$  dose was only half as effective as the 10  $\mu\text{M}$  dose at inhibiting PGE<sub>2</sub> production.

The relative ability to suppress prostaglandin synthesis of three S-oligonucleotides, mCOX-2.2, mCOX-2.3, and mCOX-2.4, was determined. Each 3T3 cell cultures received 5  $\mu\text{M}$  of one of the S-oligonucleotides for 24 hours, followed by a 4-hour stimulation period with 30 nM PMA. Control cells received 0.01% vehicle, dimethylsulfoxide, also referred to herein as "DMSO". The results are shown in Figure 3 which reflects a representative experiment and is the mean of 8 replicates per condition. The data are expressed as the % of control PGE<sub>2</sub> production.

As shown in Figure 3, the basal PGE<sub>2</sub> production by unstimulated cells is shown on the horizontal line. The PMA treatment alone stimulated a 360% increase in PGE<sub>2</sub> synthesis in the 3T3 cells relative to basal levels in untreated cells. When control cells were pretreated with S-oligonucleotide, however, there was a complete suppression of basal PGE<sub>2</sub> production as

shown by open bars in Figure 3. In PMA-treated cells there was also a complete suppression of PMA-stimulated PGE<sub>2</sub> production.

The ability of the antisense nucleotides mCOX-2.2 and mCOX-2.3 were also determined using the above culture system. Varying  
5 doses as listed in Table 5 of each nucleotide were administered to the cultures. The resulted are presented in Table 5.

Table 5

Dose-dependent Suppression of PGE<sub>2</sub> Production  
in Mouse 3T3 Fibroblasts by mCOX-2.2 and mCOX-2.3

|    | PMA     | Antisense<br>Oligonuc.<br>mCOX-2.2 | PGE <sub>2</sub><br>(pg/well) |  |          | PGE <sub>2</sub><br>(pg/well) |
|----|---------|------------------------------------|-------------------------------|--|----------|-------------------------------|
| 5  | Control | -                                  | 23±4                          |  | Control  | 25.8±0                        |
|    | Control | yes                                | 1,360±32                      |  | PMA      | 1,410±104                     |
|    |         |                                    |                               |  | mCOX-2.3 |                               |
|    | Treated | yes                                | 40 μM                         |  | 40 μM    | *                             |
|    | Treated | yes                                | 20                            |  | 20       | 302±8.5                       |
| 10 | Treated | yes                                | 10                            |  | 10       | 387±10.9                      |
|    | Treated | yes                                | 5                             |  | 5        | 453±71.3                      |
|    | Treated | yes                                | 1                             |  | 1        | 877±45.3                      |
|    | Treated | yes                                | 0.1                           |  | 0.1      | 1,075±152                     |
|    |         |                                    |                               |  |          |                               |

\* - below detectable limit. The limit of detection for the radioimmunoassay was <1.56 pg/100 μl.

As shown in Table 5, both the mCOX-2.2 and mCOX-2.3 completely suppress the prostaglandin formation at doses of 40 μM, and significant suppression occurs with 10 μM. Again, the dose dependent relationship is apparent.

The relative efficacy of the unmodified, N-oligonucleotide in comparison to phosphorothioate derivative, S-oligonucleotide was determined for the inhibition of mCOX-2 and suppression of PGE<sub>2</sub> production in 3T3 cell cultures. The cultures were prepared as described above and 10 μM of either the S-oligonucleotide or the N-oligonucleotide were added. The cultures were then exposed to 30 nM PMA.

As shown in Figure 4, the mCOX-2.4 antisense S-oligonucleotide was more effective than the unmodified S-oligonucleotide targeted against the same mRNA. The PGE<sub>2</sub> production in the S-oligonucleotide treated cells was reduced by

52% of the level of control cells, while the N-oligonucleotide treated cells reduced PGE<sub>2</sub> production by 43% of control levels. Neither the S-oligonucleotide nor N-oligonucleotide treated cells appeared to differ from control cells with respect to cellular viability as assessed by trypan blue exclusion. In addition, the basal level synthesis of PGE<sub>2</sub> was completely abolished in 3T3 cells when treated with S-oligonucleotide mCOX-2.4.

#### Cyclooxygenase Inhibition In Amnion-Derived Cells

Four human antisense S-oligonucleotides directed to different regions of the human inducible COX mRNA designated hCOX-2 were evaluated for their ability to suppress PGE<sub>2</sub> production in PMA stimulated amnion-derived WISH cells. The WISH cells are an accepted a model system for the production of PGE<sub>2</sub> in the setting of preterm labor. See: Mitchell, M.D, et. al "Immunologic Aspects of Preterm Labor", Seminars in Perinatology, 1991; 15:210-224; Kniss, D.A. et. al, "Evidence of a Role for Protein Kinase C in Epidermal Growth Factor-Induced Prostaglandin E<sub>2</sub> Synthesis in Amnion Cells," Am. J. Obstet. Gynecol., 1990; 163: 1883-1890. It appears that the amnion cell is a major contributor to the PGE<sub>2</sub> that is involved in the onset of labor at term and in preterm labor. WISH cells are an immortalized cell line derived from a normal human amnion membrane and retains nearly all of the properties of human amnion cells maintained in primary culture. The cells were cultured in Dulbecco's Modified Eagle Media/Ham's F12 Media (1:1) supplemented with 10% newborn calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 µg/ml gentamicin sulfate. The cells were grown at 37°C in 5% CO<sub>2</sub>/95% air. For the antisense experiments, the WISH cells were seeded into 48-well plates at 2 x 10<sup>5</sup>/well in the culture medium

described above and grown for 1 day at 37°C in 5% CO<sub>2</sub>/95% air. WISH cells were preincubated for 18 hours with 5 µM of either hCOX-2.1, hCOX-2.2, hCOX-2.8, or hCOX-2.9 antisense S-oligonucleotides. The cells were then challenged for 4 hours  
5 with 30 nM PMA. PGE<sub>2</sub> production was measured by specific radioimmunoassay.

Figure 5 shows that all four S-oligonucleotides effectively inhibited PMA-induced PGE<sub>2</sub> synthesis with the following order of efficacy: hCOX-2.1>hCOX-2.8>hCOX-2.9>hCOX-2.2.

10 The hCOX-2.9 antisense S-oligonucleotide directed to hCOX-2 was evaluated in a dose-response study in which varying doses of from 0.1 to 40 µM of hCOX-2.9 were added to the wish cell cultures. The results are shown in Figure 6.

As shown in Figure 6, the antisense S-oligonucleotide hCOX-  
15 2.9 produced a greater than 50% suppression of PGE<sub>2</sub>. A maximal suppression was seen at 10 µM. Moreover, the antisense S-oligonucleotides completely suppressed basal PGE<sub>2</sub> synthesis.

To verify that the inhibition by the antisense oligonucleotide was specific rather than non-specific, a sense  
20 S-oligonucleotide corresponding to hCOX-2.9 was tested in the WISH cell. There was no statistically demonstrable inhibition of WISH cell PGE<sub>2</sub> production by the sense S-oligonucleotide unless high concentrations were used (≥40 µM), at which point there was modest inhibition of PGE<sub>2</sub> biosynthesis.

25 To determine whether antisense S-oligonucleotides directed against human COX-2 mRNA alter the expression of COX-1, enzyme amnion-derived human WISH cells were plated into 35 mm dishes (2 x 10<sup>6</sup>/dish) in F12\Dulbecco's Modified Eagle Media supplemented with 10% newborn calf serum. After 24 hours, the cells were

incubated in serum-free medium for 18 hours with 10  $\mu$ M of the antisense S-oligonucleotide hCOX-2.1. Control cells received vehicle only during the preincubation. The cells were then challenged with 10 ng/ml of interleukin-1 $\beta$  for 4 hours, extracted with 1% Triton X-100 and the proteins were fractionated on a 10% polyacrylamide gel. Proteins were transferred to nitrocellulose and then probed with an antibody specific for COX-1. The western blot is shown in Figure 7.

As shown in Figure 7, the S-oligonucleotide hCOX-2.1, which suppresses the expression of the COX-2 protein, does not suppress the expression of the COX-1 protein. Thus, the antisense nucleotides specifically bind the designated mRNA.

#### Cyclooxygenase Inhibition in Endometrial Stromal Cells and Amnion Derived Cells

To determine whether antisense S-oligonucleotides directed against hCOX-1 inhibit PGE<sub>2</sub> formation, WISH cells and HES cells were used. Human endometrial stromal (HES) cells were used as a model system for the production of PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  by the uterine decidua in the setting of preterm labor, menstrual cramping and endometriosis. The human pregnancy endometrium (decidua) lining the uterine cavity and in contact with the placenta is a major contributor to the PGF<sub>2 $\alpha$</sub>  which is responsible for myometrial contractions during labor. The HES cells were developed as a model system in which to study the uterine contribution to PG production. This cell line was prepared following a uterine biopsy obtained from a woman undergoing a hysterectomy for non-malignant uterine disease. The endometrial surface was removed with a scalpel and dissociated into a single-cell suspension with trypsin. The cells were grown in

medium 199 supplemented with 10% fetal bovine serum and 50 µg/ml gentamicin. The cultures were split once per week until a single cell type exhibiting epithelial properties remained. The cell line obtained from this biopsy specimen has retained many of the properties of human endometrial/decidual cells maintained in primary culture. The cells were grown at 37°C in 5% CO<sub>2</sub>/95% air. For the antisense oligonucleotide evaluations, the cells were seeded into 48-well plates at 2 x 10<sup>5</sup>/well in the culture medium described above and grown for 1 day at 37°C in 5% CO<sub>2</sub>/95% air. The cultures were preincubated for 18 hours either with 0, 1, or 10 µM hCOX-1.1, followed by a 4 hour stimulation with 10 ng/ml interleukin-1β. PGE<sub>2</sub> synthesis was measured by specific radioimmunoassay. Antisense S-oligonucleotide hCOX-1.1 inhibited WISH cell and HES cell PGE<sub>2</sub> synthesis in a dose-dependent fashion as shown in Figure 8. However, hCOX-1.1 appeared somewhat more effective in WISH cells than HES cells. This may be due, in part, to the much greater synthesis of PGE<sub>2</sub> by HES cells when stimulated by IL-1β when compared to WISH cells.

#### Suppression of COX-2 protein in amnion-derived WISH cells.

Amnion-derived human WISH cells were plated into 35 mm dishes (2 x 10<sup>6</sup>/dish) in F12/Dulbecco's Modified Eagle Media supplemented with 10% newborn calf serum. After 24 hours, the cells were incubated in serum-free medium for 18 hours with either 5, 10, 20 or 40 µM of the antisense S-oligonucleotide hCOX-2.1. Control cells received only vehicle during the preincubation period. The cells were then stimulated for 4 hours with interleukin-1β and proteins were extracted with 1% Triton X-100, fractionated by polyacrylamide gel electrophoresis, and



blotted onto nitrocellulose membranes. The membranes were probed with rabbit polyclonal antibodies to mouse COX-2 and were subsequently detected by incubation with goat anti-rabbit IgG conjugated to alkaline phosphatase followed by development with NBT/BCIP. The western blot shown in Figure 9 has Lane 1, control; lane 2, IL-1 $\beta$  alone; lane 3, IL-1 $\beta$ +5  $\mu$ M S-oligonucleotide; lane 4, IL-1 $\beta$ +10  $\mu$ M S-oligonucleotide; lane 5, IL-1 $\beta$ +20  $\mu$ M S-oligonucleotide; and lane 6, IL-1 $\beta$ +40  $\mu$ M S-oligonucleotide.

As shown in Figure 9 the S-oligonucleotide hCOX-2.1 decreased the expression of the COX-2 protein (lanes 3-6) compared to cells treated with interleukin-1 $\beta$  alone (lane 2).

Suppression of endotoxin-induced COX-2 mRNA expression in mouse peritoneal macrophages.

Mouse macrophages were plated into 6-well plates (3 x 10<sup>6</sup>/well) in Dulbecco's modified Eagle's medium + 10% fetal bovine serum. After 24 hours, the cells were incubated in serum-free medium for 18 hours with 10  $\mu$ M of the antisense S-oligonucleotide mCOX-2.2. Control cells received vehicle only during the preincubation. The cells were then challenged with 10  $\mu$ g/ml of LPS (lanes 2 and 3) for 4 hours and total RNA was prepared and fractionated on a 1% agarose gel. After blotting the RNAs onto nylon membrane they were probed with a [<sup>32</sup>P] labeled cDNA probe encoding the murine COX-2 cDNA. The northern invention blot is shown in Figure 10.

As shown in Figure 10, there is a nearly complete elimination of COX-2 mRNA expression in LPS cells pretreated with antisense S-oligonucleotide, shown in lane 3, compared with control cells which were treated with LPS, shown in lane 2.

Thromboxane A<sub>2</sub> Synthase Inhibition in Placental Cells

To determine whether antisense S-oligonucleotides directed against the mRNA encoding human thromboxane A<sub>2</sub> synthase (TxS), inhibited production of thromoxane, the placental cell line ED<sub>27</sub> was used as a model system. The ED<sub>27</sub> cells were established as an immortalized cell line of trophoblasts isolated from human first trimester chorionic villi. These cells retain nearly all of the properties of human trophoblasts maintained in primary culture and have served as an excellent model for studies of placental cell physiology. These cells make large amounts of PGE<sub>2</sub>, PGF<sub>2α</sub>, and thromboxane in response to a variety of cytokines including interleukin-1β.

The cells were grown in F12/DMEM supplemented with 15% fetal bovine serum, glutamine, pyruvate and gentamicin at 37°C in 5% CO<sub>2</sub>/95% air. Cells were cultured as described above and preincubated for 24 hours with the antisense S-oligonucleotide hTxS-6 at doses ranging from 0.25-40 μM. Following the preincubation period, the cells were stimulated with 10 ng/ml interleukin-1β for 24 hours at which time the media were collected and thromboxane production was measured by measuring the stable metabolite, TxB<sub>2</sub>, by specific radioimmunoassay.

As shown in Figure 11, S-oligonucleotide hTXS-6 significantly inhibited the production of the TXA synthase. Furthermore there was a dose-dependent inhibitory effect of the antisense S-oligonucleotide directed against human TxA<sub>2</sub> synthase.

The antisense nucleotides of the present invention are effective at suppressing cyclooxygenase products such as prostaglandins and thromboxane and are useful in the study and treatment of preterm labor, preeclampsia, PROM, premature

cervical effacement and dilation, and endometriosis and menstrual cramping, known as dysmenorrhea.

The antisense oligonucleotides are also suitable for treating disease controlled by prostaglandins such as rheumatoid  
5 arthritis, adult respiratory syndrome, and glomerulonephritis.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Kniss, Douglas A.
- (ii) TITLE OF INVENTION: Antisense Oligonucleotides to Suppress Eicosanoid Formation
- (iii) NUMBER OF SEQUENCES: 25
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Mary E. Golrick
  - (B) STREET: 800 Superior Ave.
  - (C) CITY: Cleveland
  - (D) STATE: Ohio
  - (E) COUNTRY: U.S.A.
  - (F) ZIP: 44114-2688
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Golrick, Mary E.
  - (B) REGISTRATION NUMBER: 34,829
  - (C) REFERENCE/DOCKET NUMBER: 18525/00107
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 216-622-8458
  - (B) TELEFAX: 216-241-0816

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA to mRNA
- (iv) ANTI-SENSE: YES

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AAAACTCCTC CCTCCAGA  
18

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iv) ANTI-SENSE: YES

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCACGAAAAC CCACATCA  
18

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iv) ANTI-SENSE: YES

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCGAAAGTGG CAAAATCA  
18

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCTGGGAGTG GATGGATG  
18

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGAGGTGGCA GCGGAGGT  
18

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGAGGAATCA ATGCTGAT  
18

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GAGCATCGCA GAGGTGGC

18

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TTGGACCCCT TTGTTTGA

18

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GACTCCGGCT CATGGCGC

18

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GTAGGCTTTTG. CTGTCTGA  
18

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 12 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CGGGCGAGCA TC  
12

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCAGCGGCGG GCAGGGCG  
18

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 15 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iv) ANTI-SENSE: YES



(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GAGCATCGCA GCGGC  
15

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGCGAGCATC GCAGCGGC  
18

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 15 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCGCGGGCGA GCATC  
15

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AGGGCGCGGG CGAGCATC  
18

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ATGACTCCTT TCTCCGCA  
18

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TTTTGGCCCC CTTATGGGGT  
20

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AACCAAGCAA ACATCACA  
18

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TTATGGGAAC CGTGCTCT  
18

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GCTTCCATCA TTCCTCTG  
18

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TAGGGCAGAT TTGGATTC

18

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GGCTTTCAAT CACTTCAG

18

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GGCTTTCAAT CACTTCAG

18

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GGCTTCCATC ATTTCTCT

18

## I Claim:

1. An antisense oligonucleotide which binds to mRNA encoding cyclooxygenase and inhibits the production of cyclooxygenase.

2. The antisense oligonucleotide of claim 1, wherein the antisense oligonucleotide is selected from the following group of deoxynucleotide sequences:

5'-GGGAGTGGATGGATGTGC-3';      5'-AAAACTCCTCCCTCCAGA-3';  
5'-CCACGAAAACCCACATCA-3';      5'-CCGAAAGTGGCAAAATCA-3';  
5'-TCTGGGAGTGGATGGATG-3';      5'-AGAGGTGGCAGCGGAGGT-3';  
5'-AGAGGAATCAATGCTGAT-3';      5'-GAGCATCGCAGAGGTGGC-3';  
5'-TTGGACCCCTTTGTTTGA-3';      5'-GACTCCGGCTCATGGCGC-3';  
5'-GTAGGCTTTGCTGTCTGA-3';      5'-CGGGCGAGCATC-3';  
5'-GCAGCGGCGGGCAGGGCG-3';      5'-GAGCATCGCAGCGGC-3';  
5'-GGCGAGCATCGCAGCGGC-3';      5'-GCGCGGGCGAGCATC-3';  
5'-AGGGCGCGGGCGAGCATC-3';      5'-ATGACTCCTTTCTCCGCA-3';      and  
5'-TTTTGGCGGGGTATGGGGT-3'.

3. The antisense oligonucleotides of claim 2 wherein the oligonucleotide has the following deoxynucleotide sequence:  
5'-GGGAGTGGATGGATGTGC-3'.

4. The antisense oligonucleotides of claim 2 wherein the oligonucleotide has the following deoxynucleotide sequence:  
5'-AAAACTCCTCCCTCCAGA-3'.

5. The antisense oligonucleotides of claim 2 wherein the oligonucleotide has the following deoxynucleotide sequence:  
5'-CCACGAAAACCCACATCA-3'.

6. The antisense oligonucleotides of claim 2 wherein the oligonucleotide has the following deoxynucleotide sequence:  
5'-CCGAAAGTGGCAAAATCA-3'.

7. The antisense oligonucleotides of claim 2 wherein the oligonucleotide has the following deoxynucleotide sequence:  
5'-TCTGGGAGTGGATGGATG-3'.

8. The antisense oligonucleotides of claim 2 wherein the oligonucleotide has the following deoxynucleotide sequence: 5'-AGAGGTGGCAGCGGAGGT-3'.
9. The antisense oligonucleotides of claim 2 wherein the oligonucleotide has the following deoxynucleotide sequence: 5'-AGAGGAATCAATGCTGAT-3'.
10. The antisense oligonucleotides of claim 2 wherein the oligonucleotide has the following deoxynucleotide sequence: 5'-GAGCATCGCAGAGGTGGC-3'.
11. The antisense oligonucleotides of claim 2 wherein the oligonucleotide has the following deoxynucleotide sequence: 5'-TTGGACCCCTTTGTTTGA-3'.
12. The antisense oligonucleotides of claim 2 wherein the oligonucleotide has the following deoxynucleotide sequence: 5'-GACTCCGGCTCATGGCGC-3'.
13. The antisense oligonucleotides of claim 2 wherein the oligonucleotide has the following deoxynucleotide sequence: 5'-GTAGGCTTTGCTGTCTGA-3'.
14. The antisense oligonucleotides of claim 2 wherein the oligonucleotide has the following deoxynucleotide sequence: 5'-CGGGCGAGCATC-3'.
15. The antisense oligonucleotides of claim 2 wherein the oligonucleotide has the following deoxynucleotide sequence: 5'-GCAGCGGCGGGCAGGGCG-3'.
16. The antisense oligonucleotides of claim 2 wherein the oligonucleotide has the following deoxynucleotide sequence: 5'-GAGCATCGCAGCGGC-3'.

17. The antisense oligonucleotides of claim 2 wherein the oligonucleotide has the following deoxynucleotide sequence: 5'-GGCGAGCATCGCAGCGGC-3'.

18. The antisense oligonucleotides of claim 2 wherein the oligonucleotide has the following deoxynucleotide sequence: 5'-GCGCGGGCGAGCATC-3'.

19. The antisense oligonucleotides of claim 2 wherein the oligonucleotide has the following deoxynucleotide sequence: 5'-AGGGCGCGGGCGAGCATC-3'.

20. The antisense oligonucleotides of claim 2 wherein the oligonucleotide has the following deoxynucleotide sequence: 5'-ATGACTCCTTTCTCCGCA-3'.

21. The antisense oligonucleotides of claim 2 wherein the oligonucleotide has the following deoxynucleotide sequence: 5'-TTTTGGCGGGGTTATGGGGT-3'.

22. An antisense oligonucleotide which binds to mRNA encoding thromboxane A<sub>2</sub> synthase.

23. The antisense oligonucleotide of claim 22 wherein the antisense oligonucleotide is selected from the following group of deoxynucleotide sequences: 5'-AACCAAGCAAACATCACA-3'; 5'-TTATGGGAACCGTGCTCT-3'; 5'-GCTTCCATCATTCTCTG-3'; 5'-TAGGGCAGATTTGGATTC-3'; 5'-GGCTTTCAATCACTTCAG-3'; and 5'-GGCTTCCATCATTTCTCT-3'.

24. The antisense oligonucleotide of claim 23 wherein the antisense oligonucleotide has the following deoxynucleotide sequence: 5'-AACCAAGCAAACATCACA-3'.

25. The antisense oligonucleotide of claim 23 wherein the antisense oligonucleotide has the following deoxynucleotide sequence: 5'-TTATGGGAACCGTGCTCT-3'.



26. The antisense oligonucleotide of claim 23 wherein the antisense oligonucleotide has the following deoxynucleotide sequence: 5'-GCTTCCATCATTCCTCTG-3'.

27. The antisense oligonucleotide of claim 23 wherein the antisense oligonucleotide has the following deoxynucleotide sequence: 5'-TAGGGCAGATTTGGATTC-3'.

28. The antisense oligonucleotide of claim 23 wherein the antisense oligonucleotide has the following deoxynucleotide sequence: 5'-GGCTTTCAATCACTTCAG-3'.

29. The antisense oligonucleotide of claim 23 wherein the antisense oligonucleotide has the following deoxynucleotide sequence: 5'-GGCTTCCATCATTTCTCT-3'.

30. A method for reducing the production of cyclooxygenase products in animals comprising the steps of:

- a. providing at least one antisense nucleotide which binds to mRNA encoding for cyclooxygenase;
- b. administering the antisense nucleotide to the animal.

31. A method for reducing the production of thromboxane in animals comprising the steps of:

- a. providing at least one antisense nucleotide which binds to mRNA encoding for thromboxane synthase;
- b. administering the antisense nucleotide to the animal.

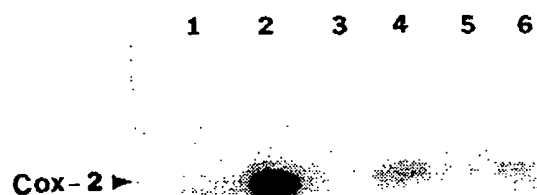


FIG. 1

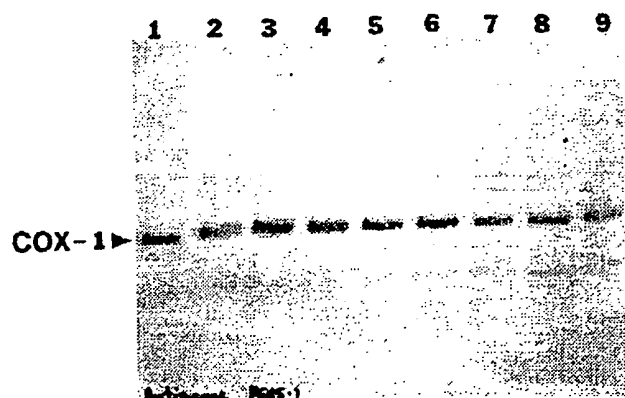


FIG. 7

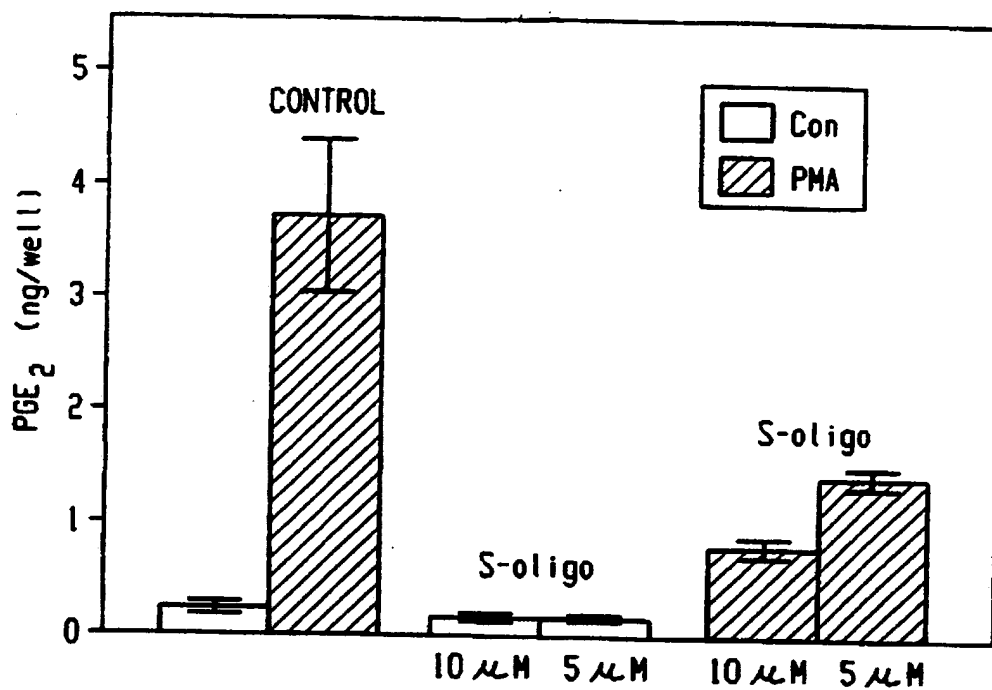


FIG. 2

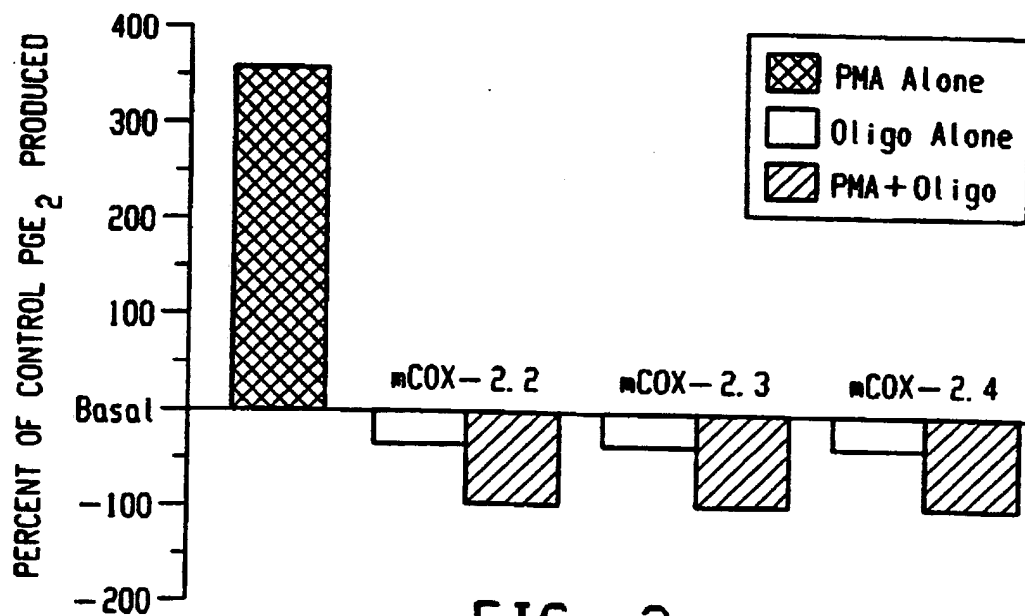


FIG. 3

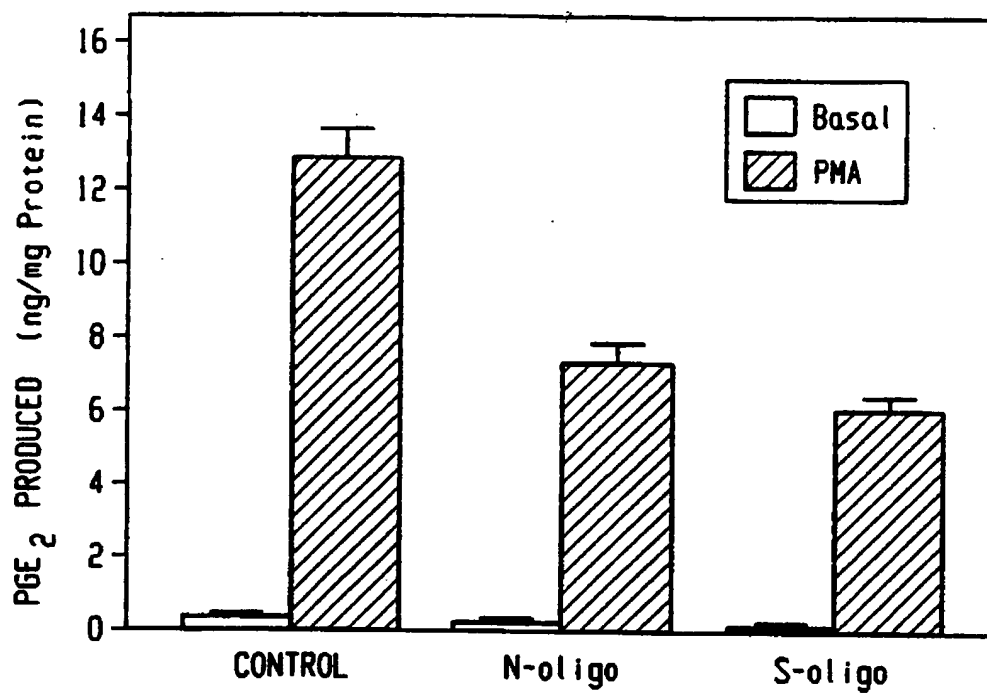


FIG. 4

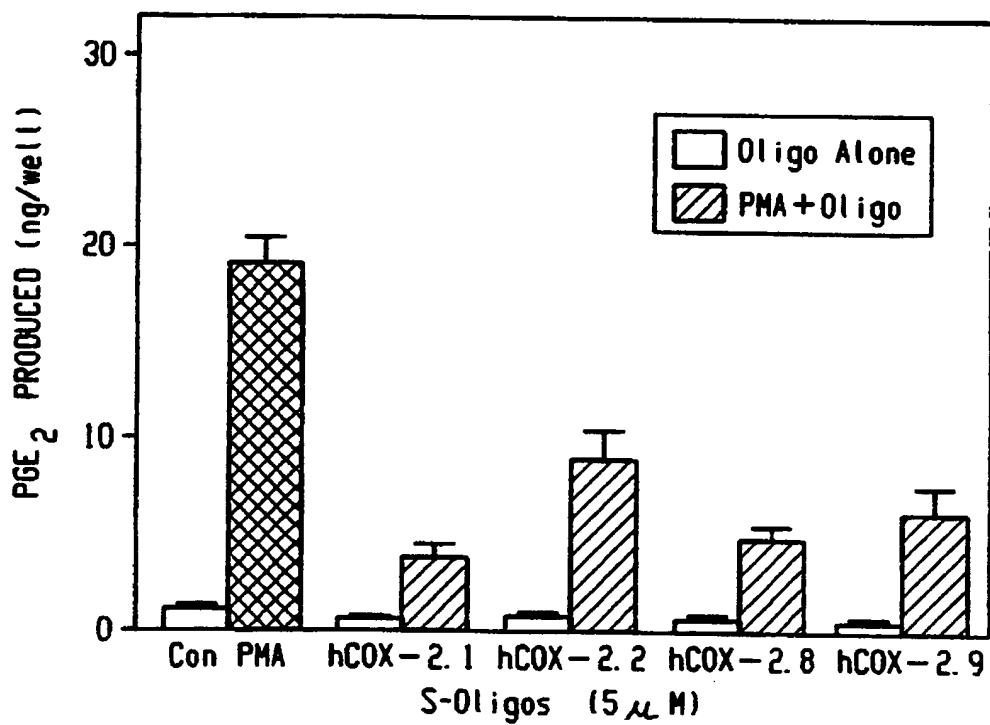


FIG. 5

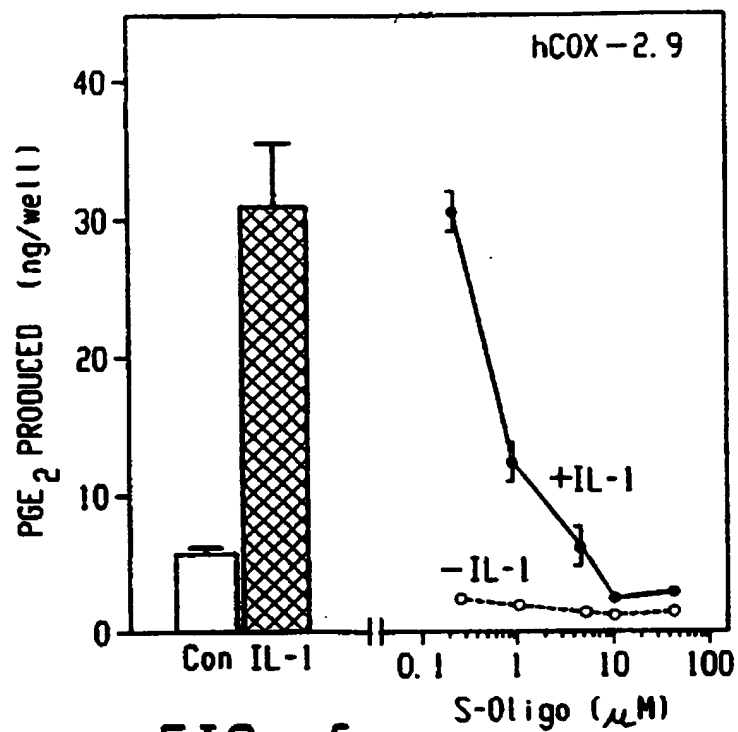


FIG. 6

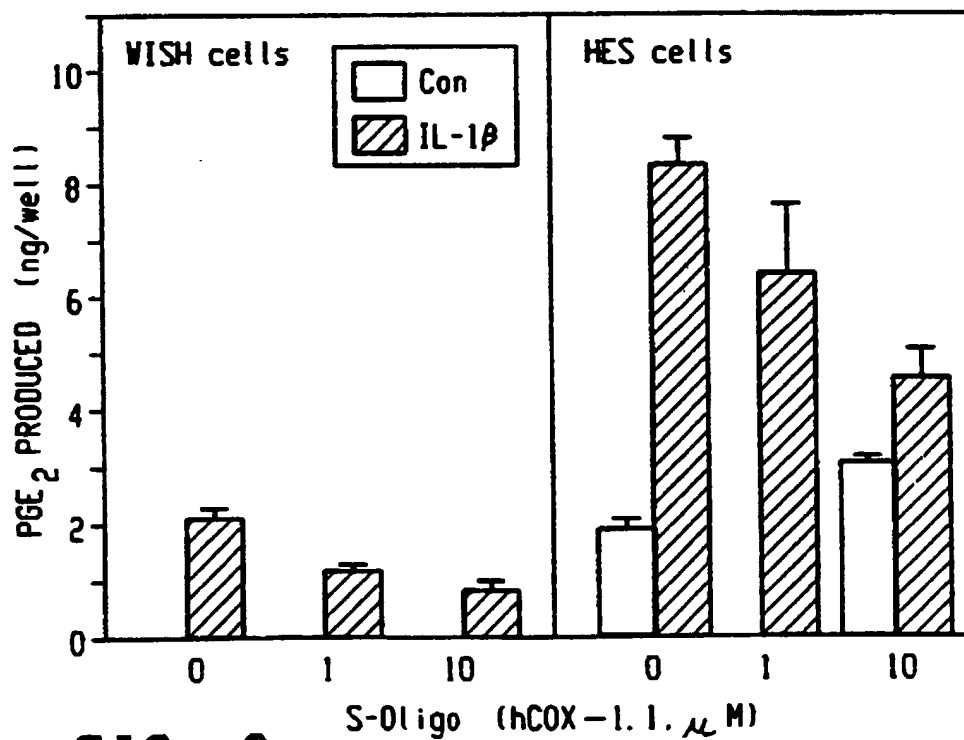


FIG. 8

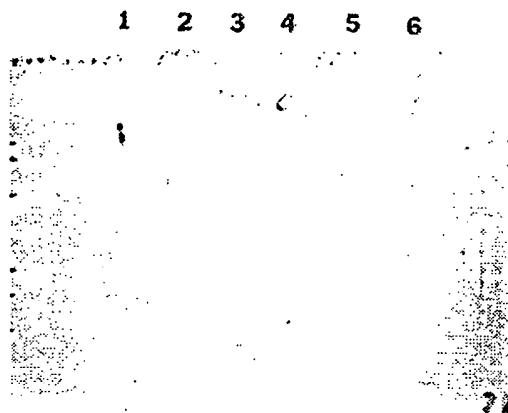


FIG. 9

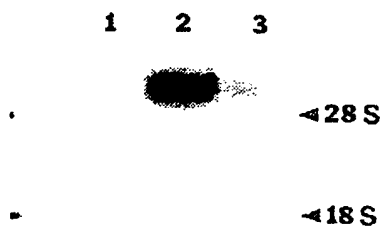


FIG. 10

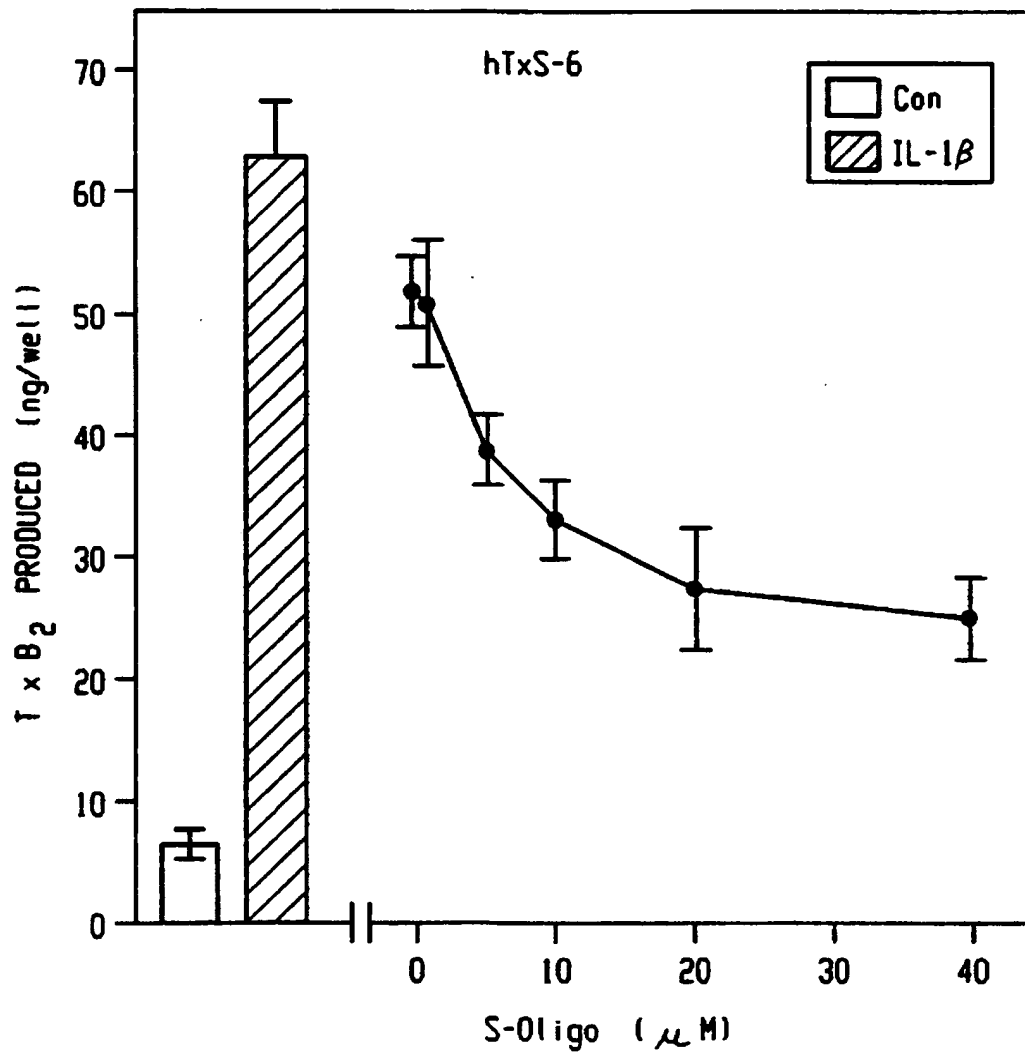


FIG. 11

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/14508

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 48/00, 31/70, 31/74; C07H 21/04; C12N 15/00, 15/11  
US CL :514/44; 536/24.5; 435/172.3

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 536/24.5; 435/172.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Derwent World Patent Index, BIOSIS

search terms: thromboxane, synthase, cyclooxygenase, antisense, inflammat?, inhib?

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|-----------|--|-----------------------|
| Y         | The Journal of Biological Chemistry, Volume 265, No. 9, issued 25 March 1990, D. DeWitt et al., "The Aspirin and Heme-binding Sites of Ovine and Murine Prostaglandin Endoperoxide Synthases", pages 5192-5196, see entire document.             | 1-7, 30               |
| Y         | Proceedings of the National Academy of Sciences USA, Volume 89, issued June 1992, M. K. O'Banion et al., "cDNA Cloning and Functional Activity of a Glucocorticoid-Regulated Inflammatory Cyclooxygenase", pages 4888-4892, see entire document. | 1, 2, 8-11, 30        |

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

|   |  |
|---|--|
| Special categories of cited documents:  |  |
| *A* document defining the general state of the art which is not considered to be of particular relevance  | *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  |
| *E* earlier document published on or after the international filing date  | *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone   |
| *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| *O* document referring to an oral disclosure, use, exhibition or other means  | *Z* document member of the same patent family  |
| *P* document published prior to the international filing date but later than the priority date claimed  |  |

Date of the actual completion of the international search

01 MARCH 1995

Date of mailing of the international search report

**24 MAR 1995**

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

CHARLES C. P. RORIES

Telephone No. (703) 308-0196



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/14508

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|-----------|--|-----------------------|
| Y         | FASEB Journal, Volume 5, issued June 1991, C. D. Funk et al., "Human Platelet/Erythroleukemia Cell Prostaglandin G/H Synthase: cDNA Cloning, Expression, and Gene Chromosomal Assignment", pages 2304-2312, see entire document. | 1, 2, 12, 30          |
| Y         | Proceedings of the National Academy of Sciences USA, Volume 89, issued August 1992, T. Hla et al., "Human Cyclooxygenase-2 cDNA", pages 7384-7388, see entire document.  | 1, 2, 13-21, 30       |
| Y         | Biochemical and Biophysical Research Communications, Volume 178, No. 3, issued 15 August 1991, C. Yokoyama et al., "Molecular Cloning of Human Platelet Thromboxane A Synthase", pages 1479-1484, see entire document.           | 22-29, 31             |
| Y         | US, A, 5,112,868 (CETENKO ET AL.) 12 May 1992, col. 19, lines 34-39, and col. 20, lines 18-45.   | 1-21, 30              |
| Y         | US, A, 5,219,874 (FAULL ET AL.) 15 June 1993, col. 1, lines 13-44.   | 22-29, 31             |
| Y         | Chemical Reviews, Volume 90, No. 4, issued June 1990, E. Uhlmann et al., "Antisense Oligonucleotides: A New Therapeutic Principle", pages 543-584, see entire document.  | 1-31                  |

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/14508

### BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

- I. Claims 1-21 and 30, drawn to antisense oligos which are complementary to cyclooxygenase (CO) mRNA, and to a method for inhibiting synthesis of CO in animals, classified in Class 514, subclass 44, for example.
- II. Claims 22-29 and 31, drawn to antisense oligos which are complementary to thromboxane synthase (TS) mRNA, and to a method for inhibiting synthesis of TS in animals, classified in Class 514, subclass 44, for example.

The claims of Groups I and II are not linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single general inventive concept as required by PCT Rule 13.1.

The oligos and method of Group I and the oligos and method of Group II have in common the technical feature of the method of using of antisense oligos to inhibit translation of a targeted mRNA of known nucleotide sequence. Hla et al. disclose the nucleotide sequence of human CO mRNA (p. 7386); and Yokoyama et al. disclose the nucleotide sequence of human TS mRNA (p. 1482). Uhlmann et al. review the use of antisense oligos as therapeutic agents capable of inhibiting expression of selected genes in a cell or organism, they teach that effective target sites for antisense oligos can potentially include any site on a targeted mRNA (pp. 569-571); they specify that antisense oligos may inhibit translation by hybridizing to mRNA at the site of translation initiation (p. 569, last paragraph), in the protein-coding sequence (p. 570, 1st paragraph), at mRNA splice sites (p. 576, left column), at or near the 5' cap region, or at the 3' end of the mRNA (p. 571, 1st paragraph; p. 576, left column); they teach that antisense oligos with as few as 13 bases (p. 545, 2nd paragraph) and as many as 48 bases (p. 576, 6th paragraph) have been shown to be effective in inhibiting RNA function; they teach that upon formation of a complex between a DNA antisense oligo and its target RNA in a cell, the RNA is cleaved and degraded by RNase H (p. 572); and they teach that antisense oligos complementary to a target site in a disease-associated RNA can be administered to an animal to inhibit metabolism of said disease-associated RNA and so provide therapeutic benefit to the treated animal (p. 577, last two paragraphs; p. 578, 1st paragraph). As the technical feature which links Group I to Group II is not an advance over the prior art, it does not constitute a special technical feature within the meaning of PCT Rule 13.2.